Activation of Stat5a And Stat5b by Tyrosine Phosphorylation Is Tightly Linked to Mammary Gland Differentiation

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Signal transducer and activator of transcription (Stat)5 was originally identified as a mammary gland factor (MGF) that binds to promoter sequences of milk protein genes and activates their transcription. We have generated isoform-specific antibodies against Stat5a or Stat5b and show that both isoforms are present in similar amounts at the protein level in mammary tissues of virgin, pregnant, lactating, and involuting mice. In contrast, Stat5 phosphorylation is very low in immature virgins, rises sharply during late pregnancy, and declines rapidly during involution. Upon phosphorylation, Stat5a and Stat5b form homo- and heterodimers. The induction of Stat5 phosphorylation during late pregnancy correlates with the transcriptional activation of milk protein genes. Using electrophoretic mobility shift assay and supershift analysis, we demonstrated that the DNA-binding activity detected during lactation is composed of both Stat5a and Stat5b, but not of other STATs. The hypothesis that Stat5 is directly involved in mammary cell differentiation was tested in estrous cycle and in transgenic mice with impaired mammary development. Transient differentiation of mammary alveolar cells and milk protein gene expression during estrus in virgin female mice coincide with transient Stat5 phosphorylation. Impaired mammary development and very low levels of milk protein gene expression in mice carrying the truncated form of the cell fate protein *Int3* correlated with reduced phosphorylation and heterodimer formation. (Molecular Endocrinology 10: 1496–1506, 1996)

INTRODUCTION

Mammary glands undergo repeated cycles of growth, differentiation, and regression. The majority of growth occurs during midpregnancy, followed by functional differentiation at late pregnancy and lactation, the hallmark of which is the expression of milk protein genes. After weaning, the glands regress and return to a resting stage through a process of extensive cell death and tissue remodeling. The presence of PRL is required for the growth and functional differentiation of mammary tissue and for the transcriptional activation of milk protein genes (1) PRL has been shown to signal through two isoforms of signal transducer and activator of transcription (Stat)5 proteins, Stat5a and Stat5b (2). Stat5 binds to promoter γ-interferon activation site (GAS; TTCNNNGAA) in the β-casein (2–4), whey acidic protein (WAP) (5), and β-lactoglobulin genes (6). Mutations in this sequence dramatically reduced the expression of milk protein genes in mammary cell lines (7) and in transgenic animals (5, 6).

Studies attempting to link mammary gland factor (MGF)-Stat5 to terminal differentiation of mammary epithelial cells and the transcription of milk protein genes have yielded inconclusive and partially conflicting results (7, 8). While the level of milk protein mRNAs in mammary tissue from immature virgins is at the threshold of detection, expression during pregnancy increases several thousand-fold and remains high throughout lactation (9). In contrast, the levels of Stat5a and Stat5b mRNA increased only slightly during pregnancy, and whereas the peak of the mRNA coincided with the onset of the milk protein gene expression, a decrease of Stat5a and 5b mRNA was observed during lactation (2, 10). Similarly, at the protein level, Stat5 showed only a slight increase during pregnancy and again a slight decrease after lactation (10). Most importantly, Stat5 is already present in mammary tissue from virgins when little milk protein gene transcription is detected (10). As an indirect measurement of activity, MGF-Stat5 was assayed by bandshift analysis (7). Very little activity was present in early pregnancy, and strong binding was detected during late pregnancy and lactation. During involution, DNA-binding activity decreased to basal levels. An-

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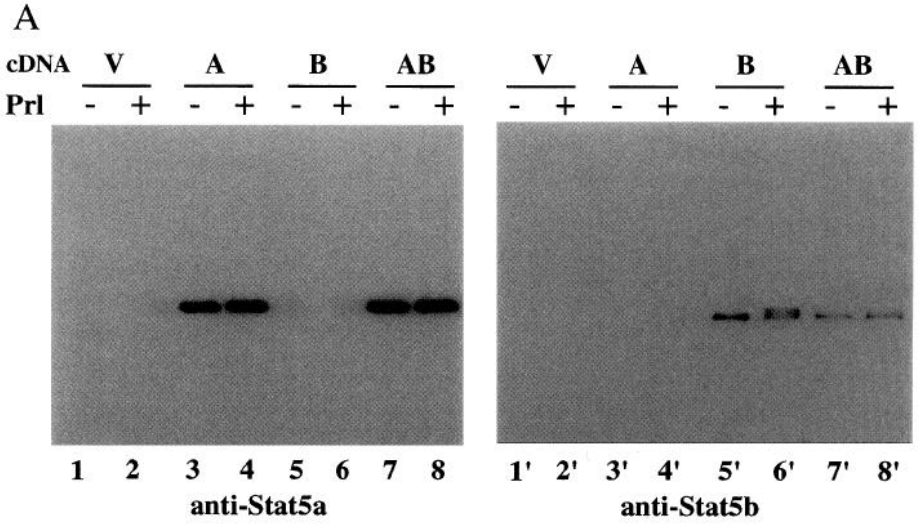
other study reported slightly different results, showing a moderate decrease in the GAS-binding activity in whole cell extracts during lactation (8). Most importantly, in neither study was the binding factor identified as MGF-Stat5 (by supershift with antibodies against MGF-Stat5, for example). This is a crucial issue because several additional STAT proteins (Stat1 through 6) have been identified, and most of them (with the exception of Stat2) have binding sites very similar or identical to the GAS sites used in the above studies (11–15).

Tyrosine phosphorylation of STATs is essential for their dimerization, translocation to the nucleus, binding to the GAS sites, and gene activation (16, 17). Phosphorylation of Stat5 at Tyr694 has been shown to be important for DNA binding and β-casein gene activation in cell culture studies (18). The presence of tyrosine-phosphorylated Stat5 proteins may provide a more physiological indicator for the activity of these proteins and provide a mechanistic explanation for the detected DNA-binding activities in the gel-shift assay. In this report, using mammary tissues derived from all stages of mammary development, including the estrous cycle, and mammary tissues from transgenic mice with impaired mammary development and differentiation, we provide evidence for a direct role of both Stat5a and Stat5b, but not other STATs, in mammary gland differentiation and milk protein expression.

RESULTS

Generation of Stat5a- and Stat5b-Specific Antibodies

Stat5a and Stat5b proteins show a 96% similarity (2), and only the C-terminal regions exhibited sufficient differences to obtain isoform-specific peptides for immunization (Fig. 1B). A unique 20-amino acid sequence of Stat5a (LDARLSPPAGLFTSARSSLS) and a unique eight-amino acid sequence of Stat5b plus three shared amino acids (MDSQWIPHAQS) were used to generate the antibodies. The specificity of the antisera was tested in COS7 cells transfected with Stat5a or Stat5b expression vectors. Western blot analysis demonstrated that anti-Stat5a antibodies recognized a protein of 93 kDa in extracts from COS7 cells transfected with a Stat5a expression vector (Fig. 1A, lanes 3 and 4) but not with Stat5b expression vector (lanes 5 and 6). Anti-Stat5b antibodies recognized a protein of 92 kDa only in extracts of COS7 cells transfected with Stat5b (Fig. 1A, lane 5′ and 6′) but not with Stat5a (lanes 3′ and 4′). Both antibodies detected the corresponding proteins in extracts of COS7 cells cotransfected with Stat5a and Stat5b (Fig. 1A, lanes 7, 8, 7′, and 8′), and neither reacted with extracts from COS7 cells transfected with the empty vector (lanes 1, 2, 1′, and 2′). PRL treatment resulted in a mobility shift



B

Stat5a veell**R**rpmd s**LDARLSPPAGLFTSARSSLS**-COOH
Stat5b veell**G**rpmd s**QWIPHAQS**-COOH

Fig. 1. Generation of Isoform-Specific Antibodies against Stat5 Proteins

A, Western blot of protein extracts from COS7 cells transfected with cDNAs encoding the long form of the murine PRL receptor and the Stat5s. V, A, B, and AB indicate transfection with the empty vector, Stat5a, Stat5b, and Stat5a plus Stat5b, respectively. Cells from each transfection were either treated with (+) or without (−) PRL. B, Isoform-specific peptide sequences used to generate the antibodies. The extreme C termini of Stat5a and Stat5b were aligned. *Bold letters* indicate amino acid residues that are different between the two. The peptides chosen for the antibody production are *underlined*.

of Stat5b due to serine phosphorylation (lanes 6′ and 8′; also H. Rui, X. L. Liu, and L. H. Hennighausen, unpublished observation). No such shift was detected for Stat5a in our gel system despite tyrosine phosphorylation (data not shown). No signal was obtained with preimmune sera (data not shown). The two antisera generated are thus isoform specific, providing important tools with which to analyze the function of each individual Stat5 isoform as well as to analyze the heterodimer formation between Stat5a and Stat5b.

Regulation of Stat5a And Stat5b during Mammary Gland Development: Changes at the Protein Level

Mouse mammary tissue contains two Stat5 isoforms, Stat5a and Stat5b (2). Kazansky and colleagues (10) reported that the level of Stat5a1 (an isoform of Stat5a) does not correlate with milk protein gene expression in rats. We assessed the protein changes of both Stat5a and Stat5b in relation to the expression pattern of milk protein genes in mice. Using isoform-specific antibodies for immunoprecipitation we evaluated the presence of Stat5a and Stat5b at different stages of mammary development. Both Stat5a and Stat5b proteins were present at an appreciable level in virgin mice during puberty (4 weeks old, Fig. 2., lane 1) and in mature virgins (6 weeks old, lane 2). A slight increase was observed during late pregnancy (lanes 6 and 7) and lactation (lanes 8 and 9), followed by a modest decrease after weaning (lanes 10–13). Direct Western blot analyses of mammary extracts provided identical results (data not shown). Clearly, the concentration of both Stat5 isoforms is highest during late pregnancy and early lactation, but the modest increase does not mirror the 100-fold increase of milk protein expression during this period.

Regulation of Stat5a and Stat5b during Mammary Gland Development: Changes in Phosphorylation

Stat5 proteins can be activated by PRL through the Janus kinase (JAK)-STAT pathway (2, 4). Ligand binding rapidly triggers tyrosine phosphorylation of STATs by the receptor-associated JAK kinases. These activated STATs dimerize and translocate into the nucleus where they bind to GAS sites and activate gene transcription. Since the transcriptional activity of STATs depends on their phosphorylation, we analyzed the

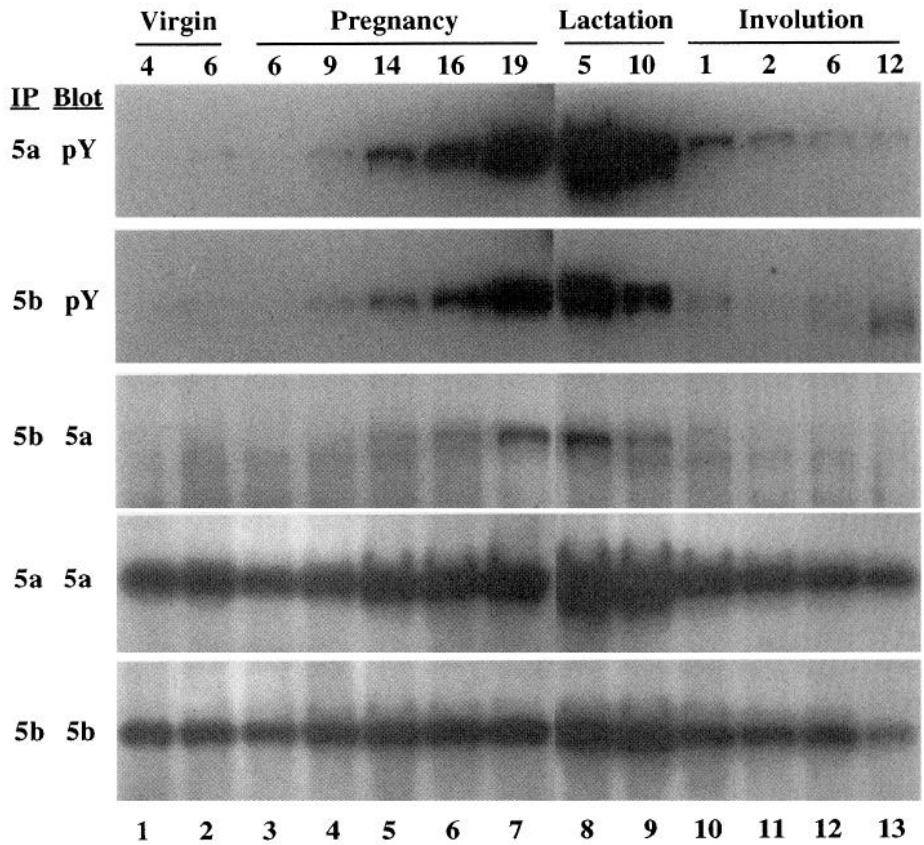


Fig. 2. Regulation of Stat5 Proteins during Mammary Gland Development

Mammary tissues were taken from immature (4 weeks) and mature (6 weeks) virgins, different stages of pregnancy (6–19 days), lactation (5 and 10 days), and involution (day 1 to 12). Immunoprecipitation (IP) was carried out using Stat5a (5a)- or Stat5b (5b)-specific antibodies and blotted with the antibodies indicated. pY represents antibody against phosphorylated tyrosine.

phosphorylation changes as a more relevant factor in relation to the pattern of milk protein gene expression.

Stat5a and Stat5b were individually precipitated from mammary tissue extracts, separated by PAGE, transferred to membranes, and probed with antibodies directed against phosphorylated tyrosine. A dramatic increase of Stat5a and Stat5b phosphorylation occurred in the second half of pregnancy (Fig. 2). The two Stat5 isoforms displayed similar kinetics of phosphorylation. Very low levels were found in virgins (lanes 1 and 2) and in early pregnancy (lanes 3 and 4), followed by a sharp increase through mid- to late pregnancy (lanes 5–7). Phosphorylation peaked during lactation (lanes 8 and 9) and decreased precipitously after weaning (lanes 10–13). Contrary to the changes of Stat5 at the level of RNAs (2, 10) and proteins (10), the pregnancy-associated increase in phosphorylation correlates closely with the expression of milk protein genes (2, 9). The weak but clearly detectable phosphorylation signal of both Stat5 isoforms observed in 6-week-old virgins (lane 2) reflects a period of transient Stat5 activation during the estrous cycle (see below).

Regulation of Stat5a and Stat5b during Mammary Gland Development: Changes in Heterodimer Formation

When induced with PRL, Stat5a and Stat5b form heterodimers in COS7 cells carrying expression vectors for Stat5a and Stat5b and the long form of the PRL receptor (data not shown). It is easy to envision that the pattern of heterodimer formation should parallel that of Stat5a and Stat5b phosphorylation. However, this correlation does not always hold true. There are instances when both Stat5a and Stat5b are phosphorylated but no heterodimer formation between the two can be detected, especially when the cells enter a state of abnormal growth (see below; also X. L. Liu, H. Rui, M. Li, G. W. Robinson, P. Furth, and L. H. Hennighausen, unpublished observations). Thus, heterodimer formation may reflect the normal physiological state of PRL signaling.

To evaluate the formation of heterodimers in mammary tissue, we probed the membrane containing Stat5b-immunoprecipited proteins with the antibody against Stat5a. Little Stat5a coimmunoprecipitated with Stat5b in mammary glands of virgin (Fig. 2, lanes 1 and 2) or early pregnant mice (lanes 3 and 4). The heterodimer formation increased in mid- to late pregnancy (lanes 5–7), peaked in early lactation (lane 8), decreased in late lactation (lane 9), and disappeared after weaning (lanes 10–13). The reciprocal experiment, *i.e.* probing the Western blot of Stat5a-immunoprecipited proteins with Stat5b antiserum, achieved the same results (data not shown). This pattern mimicked the changes in Stat5a and Stat5b phosphorylation during mammary gland development.

Regulation of Other STATs during Mammary Gland Development

The striking changes in Stat5a and Stat5b phosphorylation during pregnancy prompted us to investigate whether other STATs show similar regulation. Seven STAT members have been identified (11–15). We focused our attention on Stat1 and Stat3 because, like Stat5, they can be activated by a variety of cytokines and growth factors. Stat1 consists of Stat1α (p91) and Stat1β (p84, an alternative splice variant of Stat1 lacking 38 C-terminal amino acids of p91; see Ref. 19). Using Stat1 specific-antibodies, we immunoprecipitated both isoforms and analyzed their phosphorylation status on Western blots (Fig. 3). Interestingly, changes in Stat1 phosphorylation were almost a mirror image of that seen for Stat5. Stat1 was phosphorylated in mammary tissue from mature virgins (lane 2), during early pregnancy (lanes 3 and 4), and after weaning (lanes 10–13). However, very little phosphorylation was seen during mid- to late pregnancy (lanes 5–7) and lactation (lanes 8 and 9). Of the two isoforms, only Stat1α (p91) was phosphorylated. The level of Stat1 proteins appears to be constant throughout mammary development (Fig. 3).

Tyrosine phosphorylation of Stat3 is essentially similar to that of Stat1, inasmuch as it also is activated outside the peak of Stat5 phosphorylation, with the exception of the high level of phosphorylation at 19 days of pregnancy (Fig. 3). The significance of Stat3 activation during mammary gland involution will be addressed further in a separate report.

Both Stat5a And Stat5b Bind to the β-Casein Promoter GAS Site

We have demonstrated that tyrosine phosphorylation of Stat5a and Stat5b, but not of Stat1 and Stat3, correlates with the pattern of milk protein gene expression, as well as the previously published changes in DNA-binding activity (7, 8). To identify unambiguously the protein factors bound to the GAS site during late pregnancy and lactation, we performed a supershift experiment using antibodies against Stat5a, Stat5b, Stat1, and Stat3 (Fig. 4). Using the same GAS site described previously (2, 4, 18) and whole cell extract of mouse mammary tissue at day 5 of lactation, we demonstrated that there is a specific DNA-binding activity bound to radioactively labeled GAS sequences (lane 2) that can be competed by an excess of unlabeled oligonucleotides of the same sequences (lane 3) but not by an excess of unlabeled oligonucleotides with two base substitutions within the GAS site (lane 4; see *Materials and Methods* for the sequences of the oligonucleotides used). The DNA-protein complex can be supershifted by both Stat5a (lane 6) and Stat5b (lane 7) antibodies but not by the preimmune sera (lane 5). Antibodies against Stat1 and Stat3 cannot shift the complex. These observations indicate that the GAS-binding activity is composed of Stat5a and Stat5b but

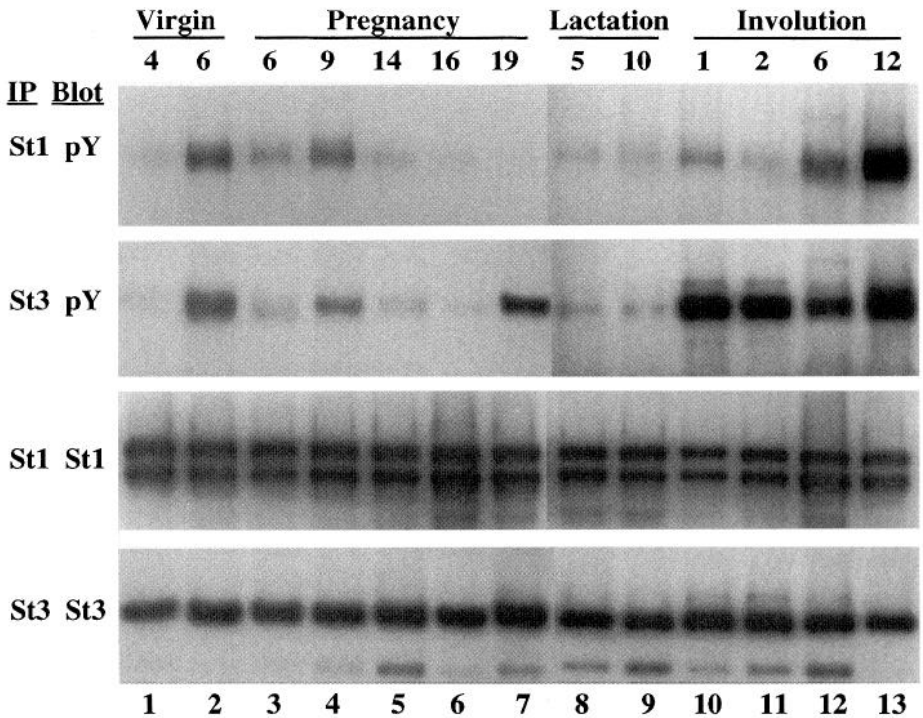


Fig. 3. Regulation of Stat1 and Stat3 during Mammary Gland Development

Immunoprecipitation was performed using antibody against Stat1 (St1) and Stat3 (St3) and blotted with antibodies as indicated. Of the two Stat1 isoforms (p91 and p84) only p91 was phosphorylated.

not of Stat1 and Stat3. It should be noted that neither antibody against Stat5 depleted the DNA-protein complex. This could be due, in part, to the fact that the antibodies used are isoform specific, and there are potentially three different Stat5 dimers complexed with the GAS sequence, Stat5a-Stat5a, Stat5a-Stat5b, and Stat5b-Stat5b.

Regulation of Stat5a and Stat5b during the Estrous Cycle

We further tested whether Stat5 phosphorylation is always correlated with mammary gland differentiation. In the absence of pregnancy, mammary glands undergo periodic limited growth and regression under the influence of ovarian steroids during the estrous cycle. A low level of milk protein gene transcription has been detected in a narrow time during the estrous cycle (20, 21). We reasoned that this transient expression of milk protein genes may also be associated with the activation of Stat5 proteins. To test this hypothesis, mammary tissues from mice at the estrous and diestrous stages were analyzed (Fig. 5). Both isoforms exist at comparable levels during the estrus (E) and diestrus (D), although at a lower level than in the lactating glands (L). However, there is a clear increase of Stat5a phosphorylation during estrus as compared with diestrus. The changes in Stat5b phosphorylation are also evident, although less dramatic. Furthermore, we found increased heterodimer formation in estrus compared with diestrus.

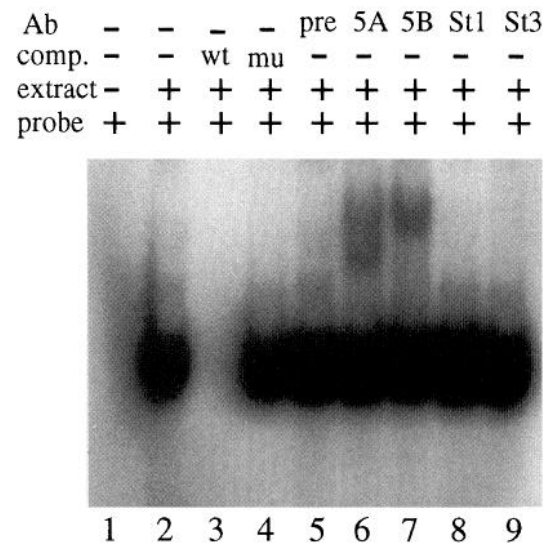


Fig. 4. Binding of Stat5a and Stat5b to the β-Casein GAS Site

Whole cell extract from mammary tissue at day 5 of lactation was incubated with labeled oligos containing the β-casein GAS site. The symbols wt and mu indicate competition with 100 × molar excess of unlabeled oligos for the binding of labeled probe to the STAT factors. The antibodies used for supershift are as follows: pre, preimmune sera of Stat5a and Stat5b; αA, αB, St1, and St3 represent antibodies against Stat5a, Stat5b, Stat1, and Stat3, respectively. The signals of free oligos are extremely strong at the bottom of the gel and have been left out of the picture.

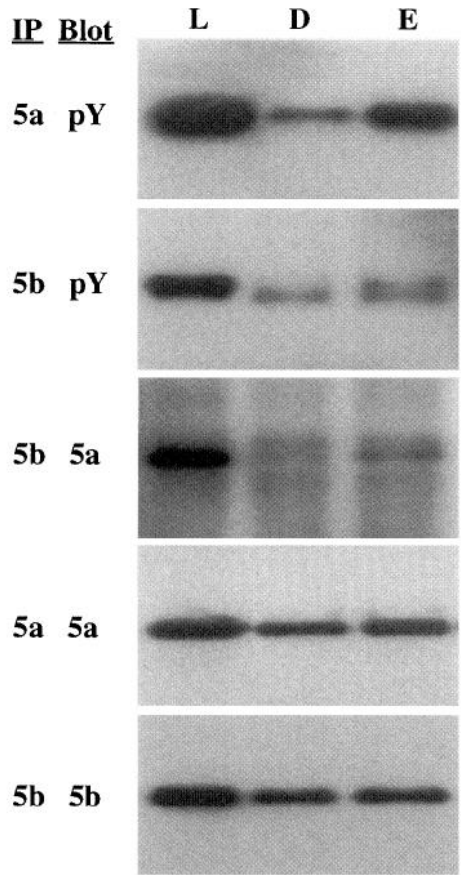


Fig. 5. Regulation of Stat5 during Estrus

Mammary tissues from 6-week-old virgin mice at estrus (E) and diestrus (D) were used in the immunoprecipitation studies as described above. Mammary tissues from mice at day 5 of lactation (L) described above were used as a positive control.

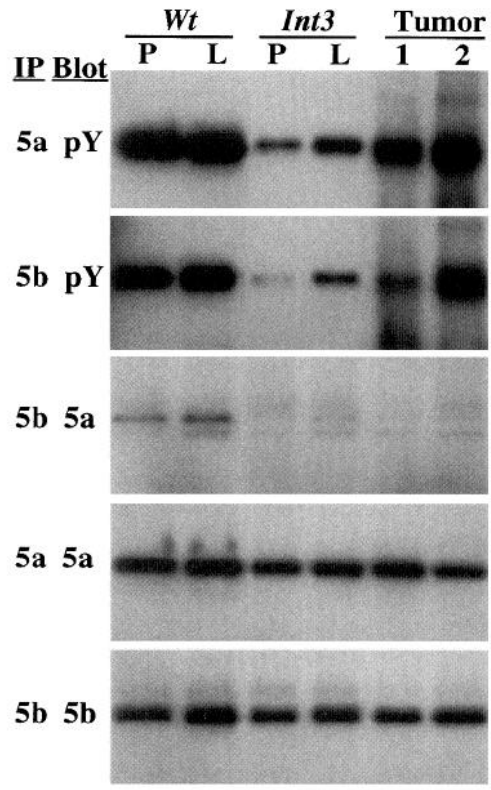


Fig. 6. Regulation of Stat5 in *WAP/Int3* Mice

Mammary tissues from WAP/*Int3* transgenic mice (*Int3*) or from wild type mice (wt) at late pregnancy (P) or after parturition (L) were used in the immunoprecipitation study as described above. *WAP/Int3* tumor tissues (Tumor nos. 1 and 2) were also included to demonstrate the uncoupling of Stat5 phosphorylation with the formation of Stat5a-Stat5b heterodimers.

Impaired Activation of Stat5a and Stat5b in Transgenic Mice That Fail to Express Milk Protein Genes

The *Int3* gene, a close relative of the *Notch* gene of *Drosophila*, is mutated in some Mouse Mammary Tumor Virus (MMTV)-induced mammary tumors in mice. Transgenic mice expressing the cytoplasmic portion of the *Int3* gene under control of the *WAP* gene promoter show impaired mammary gland development and function (22). Ductal outgrowth in transgenic virgin females is normal, but lobulo-alveolar development is severely curtailed during pregnancy. Females are unable to lactate, and little or no β-casein and WAP RNAs are found at parturition (22). To investigate whether the inability to transcribe milk protein genes was correlated with a reduced phosphorylation status of Stat5a and Stat5b, we analyzed mammary tissues from *WAP/Int3* transgenic mice at late pregnancy and after parturition (Fig. 6). Stat5a and Stat5b were present at levels comparable to nontransgenic controls. However, phosphorylation was greatly reduced. In addition, little heterodimer formation was detected in *WAP/Int3* mammary glands.

To demonstrate that heterodimer formation between Stat5a and Stat5b does not always parallel their phosphorylation, we chose to analyze two tumor tissues induced by the *WAP-Int3* expression (Fig. 6). Although Stat5b phosphorylation in tumor 1 is reduced, both Stat5a and Stat5b appear to be phosphorylated to a similar level in tumor 2 compared with those in the wild type tissue. However, no heterodimer formation can be detected in these tumor tissues.

DISCUSSION

In this report we have provided evidence that the phosphorylation of Stat5a and Stat5b is tightly linked to mammary gland differentiation and milk protein gene expression. Whereas the Stat5 messages decrease during lactation (2) and Stat5 proteins are present constitutively throughout mammary development (Ref. 10 and this report), the phosphorylation pattern of Stat5 clearly coincides with the transcrip-

tional activity of milk protein genes. Therefore, the regulation of Stat5 activation during mammary gland development appears to work at multiple levels, encompassing the regulation of the production and stability of both RNA and protein, as well as posttranslational protein modifications. This report focuses on the changes in Stat5 protein activation via tyrosine phosphorylation. As presented in our working model (Fig. 7), tyrosine phosphorylation of both Stat5 isoforms is very low in virgins and during early pregnancy, increases during mid- to late pregnancy, and reaches a plateau during lactation, followed by a sharp decrease during involution. Although GAS-binding activity during mammary gland development had been measured by other groups, the protein(s) bound to the GAS site had not been clearly identified (7, 8), partly because all STAT members, with the exception of Stat2, recognize a similar target sequence (11–15). As shown in this report (Fig. 3), the changes in phosphorylation of other STATs do not follow the same path as Stat5. Stat1 and Stat3 are tyrosine phosphorylated mostly outside of the stage of lactation. We demonstrated unambiguously that only Stat5a and Stat5b antibodies can supershift the DNA-protein complex during lactation (Fig. 4). The decreased DNA-binding activity observed during late lactation by Welte *et al.* (8) using whole cell extract seems to be consistent with our analysis of changes of Stat5 phosphorylation during late lactation, where a slight decrease of phosphorylation was detected. Therefore, the discrepancy between the two previous reports (7, 8) is probably due to changes in nuclear localization of activated Stat5 proteins. Although tyrosine phosphorylation of STAT proteins is usually rapid and transient in response to cytokine treatment (11–15), the activation of Stat5 in mammary tissue seems to be persistent and sustained, lasting from midpregnancy to the end of lactation. The changes in activated Stat5 clearly support a direct role in mammary gland differentiation and milk protein gene expression.

Signaling Molecules Activating Stat5

Stat5 has been shown to be activated by many cytokines and growth factors, including interleukin (IL)-3 (23–25), IL-5 (24), IL-6 (26), granulocyte-macrophage colony-stimulating factor (24, 27), colony-stimulating factor (28), interferon-γ (28), erythropoietin (23, 27, 29–31), GH (27, 32–35), thrombopoietin (36, 37), IL-2 (29, 38–41), IL-7 (42, 43), IL-15 (40), laminin-1 (44), epidermal growth factor (EGF) (45), and PRL (2, 4, 10, 18, 23, 27, 39). Among these, PRL and EGF have been shown to participate in the development of mammary epithelial cells; EGF exerts its effect during the proliferation phase and PRL during differentiation (see Refs. 46–51 for reviews). This makes PRL the most likely signal for Stat5 activation. The level of PRL receptor (PRLR) has been shown to be relatively constant during the pregnancy-lactation cycle in mice (52), although in rats there is an increase in PRLR RNA during lactation (53) and a 5-fold increase in PRL-binding activity (54). The level of serum PRL does fluctuate throughout the pregnancy-lactation cycle. However, PRL changes do not follow an identical pattern of Stat5 phosphorylation. Concentration of PRL in serum is high in early pregnancy, much higher during lacta-

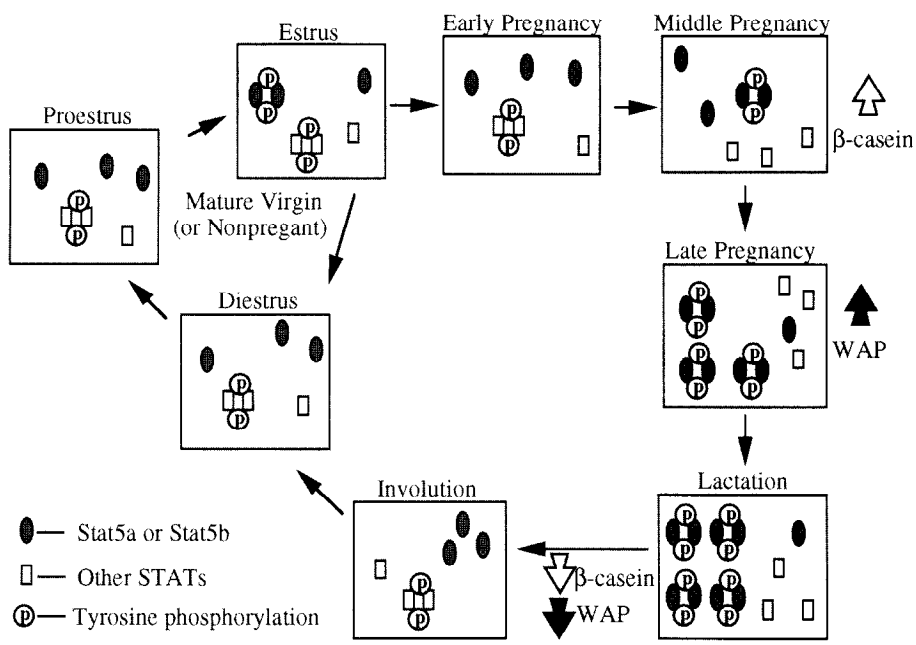


Fig. 7. A Working Model for STAT Protein Activation during Mammary Gland Development

The *open and filled wide arrows* represent β-casein and WAP, respectively; the *upward arrow* indicates on and the *downward one* indicates off. See text for more details.

tion, and very low in late pregnancy (reviewed in Ref. 55). There are several possible, but not mutually exclusive, explanations for this discrepancy. First, the placental lactogenic hormones PL-1 and PL-2 have been shown to signal through the PRLRs (reviewed in Ref. 56). Most noticeably, PL-2 appears first at midpregnancy and peaks at or near parturition in mice (57). Second, it has been shown that PRL is synthesized by mammary tissue (58–61), which may result in an autocrine or paracrine activation of Stat5. We are currently performing experiments addressing these questions.

Stat5 and Functional Mammary Differentiation

Several lines of evidence link Stat5 activation to the functional differentiation of mammary epithelial cells. The phosphorylation and activation pattern of Stat5a and 5b parallels the transcription of milk protein genes, the transient differentiation of epithelial cells during estrus coincides with Stat5 phosphorylation, and the inability of mammary cells of *WAP/Int3* transgenic mice to develop and terminally differentiate coincides with decreased Stat5 activation. The JAK-STAT pathway is widely used by cytokines and growth factors; some promote cell proliferation, others promote differentiation, and some have both properties, depending on the target cells and the physiological state of the cells (11–15). In the mammary system, based on the evidence provided here, Stat5 appears to be a differentiation factor. The peak of phosphorylation correlates with the terminal differentiation of the glands. In contrast, Stat5 proteins are mostly unphosphorylated at the time of massive proliferation of the mammary epithelial cells (early to midpregnancy). Stat5-like factors have been shown to be activated by cytokines such as colony-stimulating factor-1 and INF-γ, which have the potential to promote differentiation in myeloid cells as well (28, 62).

Furthermore, the ability of Stat5a and Stat5b to form heterodimers, in addition to homodimers, may distinguish the differentiated from a nondifferentiated cell. Heterodimerization between Stat5a and Stat5b occurs whenever the analyzed cell has acquired a state of differentiation, both during lactation and in estrus. The Stat5 isoforms are virtually identical in their SH2 domain, and it is therefore very likely that they are recruited to the same docking site (phosphorylated tyrosine residue) on a receptor complex. Depending upon the recruitment of Stat5 isoforms to the paired receptor molecules, Stat5a and Stat5b can form either homo- or heterodimers. We have observed the failure of Stat5a and Stat5b to form heterodimers in defined circumstances, even though both isoforms are phosphorylated. In particular, the inability of phosphorylated Stat5a and Stat5b to form heterodimers in the nondifferentiated mammary tissue of postpartum *WAP/Int3* transgenic mice and in *WAP/Int3* tumors clearly supports the notion that heterodimerization may be necessary for functional differentiation. We postulate that Stat5 heterodimer formation reflects the normal physiological state of PRL signaling, and these molecules have distinct functions that cannot be performed by the respective homodimers. Because tissue culture cells do not permit the study of the wide range of Stat5 signaling, this hypothesis will be tested in mice in which either one of the Stat5 genes has been deleted from the mouse genome. We are currently generating Stat5a and Stat5b knockout mice to pursue these possibilities.

MATERIALS AND METHODS

Plasmid Construction and COS Cell Transformation

Plasmid pcDNA-5A was constructed by cloning a 3.6-kb cDNA encoding 50 bp of 5′- and 1.2 kb of 3′-untranslated sequences plus 2.4 kb of the coding region of Stat5a cDNA (2) into the *Eco*RI/*Xba*I-blunt sites of the expression vector pcDNAI/Amp (Invitrogen, San Diego, CA). Similarly, Stat5b expression construct pcDNA-5b was made by cloning into the pcDNAI/Amp vector a 5.0-kb fragment containing 52 bp of 5′- and 2.6 kb of 3′-untranslated region plus 2.4 kb of protein-coding sequence. The plasmid encoding the long form of the rat PRL receptor has been described previously (10) and was kindly provided by Ms. Lindsay and Dr. Rosen.

COS 7 cells were cultured in DMEM medium supplemented with 10% FCS in a 37 C incubator with 5% CO_2. Transfection was done by electroporation using a Gene-Pulser (Bio-Rad, Richmond, CA) at the setting of 220 V and 960 μFarads; 5×10^6 cells in 250 μl of medium with 5 mM *N,N*-bis(2-hydroxylethyl)-2-aminothanesulfonic acid were mixed with 5 μg PRLR cDNA plus 5 μg pcDNA-5A and/or 5 μg pcDNA-5B, or 5 μg of the vector pcDNAI/Amp (made up to 40 μg of total DNA with salmon sperm DNA) and electroporated. Transfected cells were incubated in DMEM plus 10% FCS. The medium was replaced 24 h later with basal medium (DMEM plus 1% of FCS) and incubated overnight. Cells were then treated with ovine PRL (Sigma, St. Louis, MO) at 5 μg/ml for 15 min before being harvested for protein extract preparation.

Antibodies

Carboxyl-terminal peptides specific for the mouse Stat5a (LDARLSPPAGLFTSARSSLS) or Stat5b (MDSQWIPHAQS, Fig. 1B; also see Refs. 2, 24, and 25) were conjugated to keyhole limpet hemocyanin and used for immunization of rabbits. The specificity of the antisera was analyzed by Western blot of protein extract of COS7 cells transfected with the corresponding cDNA expression vectors for Stat5a and Stat5b. Antibodies against Stat1 and Stat3 were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA).

Western Blotting

COS7 cell extracts or mammary extracts were resolved on precasted SDS-PAGE using the mini-gel system (Novex, San Diego, CA). Proteins were then transferred to PVDF membranes using the transfer module as instructed by the manufacturer (Novex). Blots were blocked overnight with TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20) plus 2% BSA. Primary antibody diluted in TBST/2% BSA was then incubated with blots for 1 h at room temperature. The antisera for Stat5a and Stat5b were diluted 1:20,000 and 1:10,000, respectively. The anti-phosphotyrosine antibody

(4G10, Upstate Biotechnology, Inc., Lake Placid, NY) was diluted to 0.4 μg/ml (1:2,500). After three washes of 5 min each with TBST, horse radish peroxidase-conjugated goat anti-rabbit (or mouse, depending on the primary antibody used) IgG (Sigma) diluted to 1:4,000 was added and then incubated for another 30 min at room temperature. Four 15-min washes were carried out, and the blots were developed using the ECL detection system (Amersham, Arlington Heights, IL). The blots were stripped by incubating in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 μM β-mercaptoethanol for 30 min, with occasional agitation at 65 C, and reblotted as described above.

Animal Studies

All wild type female C57/Bl6 mice were obtained from the mouse facility at the National Cancer Institute (Frederick, MD). The fourth pair of mammary glands were taken at different stages of mammary development, starting from 4 weeks (puberty), 6 weeks (mature), various stages of pregnancy, lactation, and involution. For mature virgin females, the glands were also taken from estrous and diestrous stages, determined by histological examination of vaginal smears. The transgenic *WAP/Int3* mice have been described previously (22).

All animal studies were conducted in accord with the principles and procedures outlined in the NIH Guidelines of "Using Animals in Intramural Research."

Immunoprecipitation of Mouse Mammary Tissue Extracts

At different stages of mammary development, the fourth pair of mammary glands from two (six for the virgin mice) C57/B6 mice were pooled and immediately frozen in liquid nitrogen. Before analysis, tissue samples were weighed and sliced with a razor blade. Eight milliliters of lysis buffer (40 mM Tris-HCl, pH 8.0, 276 mM NaCl, 20% glycerol, 2% NP-40, 4 mM EDTA, 20 mM sodium fluoride, 2 mM sodium orthovanadate, 40 μg/ml phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 50 μg/ml leupeptin) were added to each gram of tissue and then homogenized using a polytron device as described before (2). The extracts were cleared by spinning in an Eppendorf centrifuge at 10,000 rpm at 4 C for 15 min.

Extracts containing 1.5 mg protein were incubated with 1 μl Stat5a or 2 μl Stat5b antisera for 30 min at 4 C on a vertical rotator. Protein A-Sepharose beads (Sigma) were added, and the incubation was continued overnight. The samples were washed three times with lysis buffer, resuspended in 2× sample buffer (250 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.006% bromophenol blue), boiled for 3 min, and centrifuged briefly. The supernatant was applied to an 8% precasted SDS-PAGE, and proteins were transferred to PVDF membranes as instructed by the manufacturer (Novex).

Electrophoretic Mobility Shift Assay (EMSA)

The probe was derived from the ovine β-casein promoter (5′-AGATTTCTAGGAATTCAAATC-3′) as described previously (2, 4, 18). Mutated oligonucleotides used for the competition contained two base substitutions changing the two G residues into two Ts (5′-AGATTTCTATTAATTCAAATC-3′), which has been shown sufficient to abolish its ability to compete with the wild type oligonucleotides (3). Mammary tissue from two mice at day 5 of lactation was homogenized in lysis buffer containing 10 mM Tris·Cl, pH 8, 1 mM EDTA, 1 mM dithiothreitol, 0.4 M KCl, 10% glycerol, 1 mM sodium vanadate, plus protease inhibitors as described in the above section. Binding reaction was carried out 30 min at room temperature in a 20-μl volume containing 15 mM Tris·Cl, pH 8, 1 mM EDTA, 100 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 1.5 mg/ml poly(deoxyinosinic-deoxycytidylic acid), 12% glycerol, with 20 μg cleared whole cell extract and 4×10^4 cpm of double-stranded DNA oligonucleotides labeled by kinasing with [γ-P^{32}]ATP. Supershift was performed by preincubating the whole cell extract with the appropriate antibodies [1 μl of Stat5a or Stat5b (1:10 dilution) or 1 μl each of preimmune sera for Stat5a and Stat5b (1:10 dilution); 1 μl of Stat1 or Stat3 from Santa Cruz) for 30 min on ice. Electrophoresis was carried out at room temperature in a 4% polyacrylamide gel containing 2.5% glycerol in 0.25 × Tris-borate-EDTA buffer.

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